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(21) International Application Number: PCT/GB98/01725 (22) International Filing Date: 12 June 1998 (12.06.98) (30) Priority Data: 9712282.4 12 June 1997 (12.06.97) GB (71) Applicant (for all designated States except US): JOHN INNES CENTRE [GB/GB]; Norwich Research Park, Colney, Norwich NR4 7UH (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): LOMONOSSOFF, George, Peter [GB/GB]; John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH (GB). TAYLOR, Kathryn, May [GB/GB]; John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH (GB). (74) Agents: KREMER, Simon, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(54) Title: POLYPEPTIDE PRESENTATION SYSTEM			
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(57) Abstract			
<p>Disclosed are nucleic acid constructs comprising a sequence encoding a plant viral coat protein (e.g. the S-peptide of CPMV) containing a foreign or heterologous peptide insert (e.g. an epitope for vaccine use) wherein the said coat protein has been modified such as to reduce its ability to effect nucleic acid packaging within viral particles. The modification is preferably at the C-terminus. Also disclosed are corresponding nucleic acid preparations, plus methods, processes and other materials (e.g. plants, virus particles, and compositions) based on the nucleic acids.</p>			

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## POLYPEPTIDE PRESENTATION SYSTEM

Technical field

5 The present invention relates to the display or  
presentation of a polypeptide (e.g. an epitope) on the  
surface of plant viral particles, useful for immunisation  
to provoke in a mammal an immune response to the epitope,  
which may be against one or more pathogens including the  
10 epitope. More particularly, the invention provides for  
production of such viral particles which lack  
encapsulated nucleic acid. Nucleic acid constructs,  
methods of production of the particles, compositions,  
uses and other aspects of the invention are provided  
15 herein.

Prior art

The use of plant viruses, particularly comoviruses such  
as cowpea mosaic virus (CPMV), as an epitope presentation  
20 system has been demonstrated previously (Porta et al.,  
(1994) Virology 202, 949-945; Usha et al., (1993)  
Virology 197, 366-374). The  $\beta$ B- $\beta$ C loop of the Small (S)  
coat protein was chosen as the site of insertion, this  
being well exposed on the surface of the particle. A  
25 variety of heterologous peptides have been expressed on  
the surface of CPMV and the resultant chimaeric particles  
have been shown to have good immunogenicity. Such  
peptides include epitopes from human rhinovirus 14 (HRV-  
14), human immunodeficiency virus type 1 (HIV-1) and  
30 foot-and-mouth disease virus (FMDV) serotype O. Recently,  
a CPMV-based chimaera displaying an epitope from mink  
enteritis virus was shown to prevent the onset of  
clinical disease in challenge experiments in mink  
(Dalsgaard et al. (1997) Nature Biotechnology 15, 248-  
35 252).

Epitope presentation on plant viruses is the subject of

prior patent applications of a present co-inventor. These applications, originally published as WO92/18618 and WO96/02649, are incorporated herein by reference as fully as if recited in full herein, primarily for their disclosure of basic technology relating to epitope display on plant viruses. The present invention is based on a new modification of the previous systems which provides at least one surprising advantage, and is useful and applicable at least in all the contexts disclosed in these previous applications, so the attention of the skilled reader is specifically drawn to the publications.

#### WO92/18618

This describes the utilisation of plant viruses as vector systems for the expression of foreign nucleotide sequences, i.e. nucleotide sequences (RNA or DNA) which are not present in plant viruses as found in Nature, and which in consequence code for peptides not normally found in any naturally occurring plant virus. The invention disclosed therein comprises assembled particles of a plant virus containing a foreign peptide. The plant viruses of the invention are therefore modified forms of the native viruses and for convenience may be referred to as modified viruses.

The foreign peptides which may be incorporated into plant viruses according to WO92/18618 and indeed the present invention may be of highly diverse types and are subject only to the limitation that the nature and size of the foreign peptide and the site at which it is placed in or on the virus particle do not interfere with the capacity of the modified virus to assemble when cultured *in vitro* or *in vivo*. In broad concept, modified viruses may be formed from any biologically useful peptides (usually fragments of polypeptides) Examples of such peptides are peptide hormones, enzymes, growth factors, antigens of protozoal, viral, bacterial, fungal or animal origin,

antibodies including anti-idiotypic antibodies, immunoregulators and cytokines, e.g. interferons and interleukins, receptors, adhesions, and parts of precursors of any of the foregoing. The peptide preferably contains more than 5 amino acids e.g. about 6-10, 10-20, or 20-30 or more. The system is highly versatile in regard to the size of the foreign peptide which may be inserted into the viral coat protein. Thus peptides containing up to 38 or more amino acids have been successfully inserted in the course of our continuing research. Preferably, the peptide insert includes an epitope to which an immune response, e.g. including an antibody response, can be raised.

Among the broad range of bioactive peptide sequences presented on plant virus vectors in accordance with WO92/18618 and the present invention special importance attaches to the antigenic peptides which are the basis of compositions which may be used to provoke an immune response in the manner of a vaccine, particularly animal (including human) virus vaccines. It should be noted that a composition which provokes an immune response may have prophylactic (i.e. protection against disease) or therapeutic (i.e. treatment of disease) applications.

WO96/02649

This discloses advantageous refinement of the peptide display technology of WO92/18618. It teaches that the process used for modifying the plant viral nucleic acid by introducing a nucleotide sequence coding for a foreign peptide should avoid the presence of direct sequence repeats flanking the insert. In such context, a construct containing a direct sequence repeat is one in which an identical oligonucleotide sequence is present on both sides of the inserted nucleotide. Such constructs can be genetically unstable because recombination can occur between the sequence repeats leading to loss of the

foreign peptide coding sequence and reversion to the wild-type sequence. It also teaches that where the foreign oligonucleotide sequence is inserted into the plant virus genome as a substitution for part of the existing sequence, the resultant modified viral coat protein may be missing in an amino acid sequence which is important for virus replication, encapsidation and spread in the plant. This defect may be readily determined and avoided.

#### *Nucleic acid in viral particles*

Comoviruses are a group of at least fourteen plant viruses which predominantly infect legumes. Their genomes consist of two molecules of single-stranded, positive-sense RNA of different sizes which are separately encapsidated in isometric particles of approximately 28nm diameter. The two types of nucleoprotein particles are termed middle (M) and bottom (B) component as a consequence of their behaviour in caesium chloride density gradients, the RNAs within the particles being known as M and B RNA, respectively. Both types of particle have an identical protein composition, consisting of 60 copies each of a large (VP37) and a small (VP23) coat protein. In addition to the nucleoprotein particles, comovirus preparations contain a variable amount of empty (protein-only) capsids which are known as top (T) component, again because of their behaviour in caesium chloride density gradients. Since the T component contains no nucleic acid it is not infectious on plants, whereas a mixture of B and M components are infectious. In a preparation of CPMV there is usually only a very small proportion of T component, less than 10% of the particles in a given preparation, generally around 5%, and it may be undetectable.

In the case of the type member of the comovirus group

(CPMV) it is known that both M and B RNA are polyadenylated and have a small protein (VPg) covalently linked to their 5' terminus. More limited studies on other comoviruses suggest that these features are shared by the RNAs of all members of the group. Both RNAs from CPMV have been sequenced and shown to consist of 3481 (M) and 5889 (B) nucleotides, excluding the poly (A) tails (van Wezenbeek *et al.*, 1983; Lomonossoff and Shanks, 1983). Both RNAs contain a single, long open reading frame, expression of the viral gene products occurring through the synthesis and subsequent cleavage of large precursor polypeptides. Though both RNAs are required for infection of whole plants, the larger B RNA is capable of independent replication in protoplasts, though no virus particles are produced in this case (Goldbach *et al.*, 1980). This observation, coupled with earlier genetic studies, established that the coat proteins are encoded by M RNA.

#### 20 *Use of chimaeric virus particles*

It can be seen from the forgoing that one limitation of the prior art systems is the presence of nucleic acid, the nucleic acid genome (RNA in the case of comoviruses such as CPMV) of the plant virus, within the chimaeric particles. Although the CPMV genome is not known to cause symptoms in animals, it is nonetheless a replicating RNA molecule. Therefore, the injection of the CPMV genome into animals (particularly at relatively high concentrations) is, for numerous safety and regulatory reasons, discouraged and the removal/exclusion of nucleic acid from the capsids would be advantageous. However the removal of RNA from wild-type CPMV particles, while keeping the capsid intact, may be difficult to achieve.

#### Disclosure of the invention

The present invention is based on the surprising discovery that manipulation of nucleic acid encoding the S capsid protein of a plant virus such as a comovirus so that the S protein lacks its natural C-terminus provides for the production of viral particles lacking nucleic acid in much greater quantities than are detected using nucleic acid encoding full-length S capsid protein. This observation can be applied generally in the production of predominantly nucleic-acid free viral particles.

It should be noted that the Small capsid protein of comoviruses is naturally proteolytically cleaved to produce a mixture of different forms with different electrophoretic mobilities. Cleavage takes place predominantly at a site towards the C-terminus. In Cowpea Mosaic Virus (CPMV) S protein 24 amino acids are cleaved from the C-terminus, the precise nature of the cleavage (whether endo- or exo- in nature) is not clear. In Bean Pod Mottle Virus (BPMV) S protein cleavage removes 9 or 13 amino acids from the C-terminus. Similar cleavage can be presumed in other members of the comovirus group, such as Andean Potato Mottle Virus (APMV), Bean Rugose Mosaic Virus (BRMV), Broad Bean Stain Virus (BBSV), Broad Bean True Mosaic Virus (BBTMV), Cowpea Severe Mosaic Virus (CPSMV), Glycine Mosaic Virus (GMV), Pea Mild Mosaic Virus (PMMV), Quail Pea Mosaic Virus (QPMV), Radish Mosaic Virus (RaMV), Red Clover Mottle Virus (RCMV), Squash Mosaic Virus (SqMV) and Ullucus virus C. See Lomonossoff and Johnson, *Prog. Biophys. molec. Biol.* Vol. 55, 107-137, 1991, for a review of comovirus synthesis and structure. An alignment of sequences of CPMV, BPMV and RCMV is given in Lomonossoff & Johnson (1991 *supra*). Sequence information on both coat proteins of cowpea severe mosaic virus (CPSMV) is given in Chen, X & Bruening, G. (1992), *Virology* 187, 682-692, and information on the S protein of Andean potato mottle virus (APMV) is given in Shindo



*et al.*, (1992), *Plant Molecular Biology* 19, 505-507.

Thus in "wild-type" viral particles, full-length S  
protein is produced on expression from the encoding  
5 nucleotide sequence within the viral genome, and  
subsequent proteolytic cleavage (e.g. on storage) results  
in production of the cleaved form lacking the original C-  
terminus, and in particular lacking a C-terminal region  
of around 30 amino acids in length, usually less than  
10 about 24 (e.g. 16-24), 13 or 9 amino acids in length.

It should be stressed that, in contrast to this  
processing of the natural or native protein, S protein of  
the various aspects of the present invention is produced  
15 only in a form lacking the natural or wild-type C-  
terminus, such as a truncated form missing a number of C-  
terminal amino acids, or amino acids within the C-  
terminal region. The present invention provides for  
production of greater quantities of substantially pure  
20 - plant viral particles lacking nucleic acid than were  
previously possible and for a simpler and more  
reproducible particle population, helpful commercially  
from the point of view of quality control etc.

25 Thus in accordance with certain aspects and embodiments  
of the present invention, modified S protein may be  
employed in generating plant viral particles lacking  
nucleic acid lacks its natural C-terminus. This may be  
by virtue of the encoding sequence of the nucleic acid  
30 used in its production being truncated to produce  
truncated protein, or by other mutation at the C-terminus  
to alter residues required for nucleic acid packaging  
within viral particles. Truncation may be at the site of  
the natural proteolytic cleavage of the C-terminus of the  
35 S protein, e.g. for CPMV so that the wild-type C-terminal  
24 amino acids are not included, and for BPMV so that the  
wild-type C-terminal 9 or 13 amino acids are not

included. Thus nucleic acid according to the present invention and employed in various aspects of the present invention may encode a plant viral capsid protein (e.g. VP23 of CPMV or other comovirus) lacking C-terminal  
5 residues which are naturally proteolytically cleaved from full-length capsid protein, i.e. terminating at the site of natural proteolytic cleavage of C-terminal residues.

Nucleic acid encoding one particular C-terminal deletion  
10 of CPMV S protein (which did not include a heterologous insert) was discussed in a poster of Taylor, Spall & Lomonossoff (Dept of Virus Research, JIC, Norwich UK, presented at the X International Congress of Virology, Jerusalem, August 1996). The authors suggested at the  
15 time that the C-terminal region may be involved in the spread of the CPMV virus. No disclosure of its relevance in nucleic acid packaging was made, or indeed envisaged, in this presentation.

20 It should be noted that in general, unless the context requires otherwise, use of the term "wild-type" herein with reference to a viral particle or capsid protein permits of mutation or variation. Generally, the term is used to distinguish from modified, e.g. truncated, capsid  
25 protein and viral particles including such capsid protein and lacking nucleic acid as disclosed, capsid protein which is not so modified and viral particles including such capsid protein not so modified, irrespective of the inclusion of one or more additional or unrelated  
30 modifications, including for example the insertion of a foreign or heterologous peptide within the capsid protein at an appropriate site as discussed.

The various aspects the present invention which will now  
35 be discussed are based on the work done by the present inventors to provide for the production and use of plant viral particles which display a foreign peptide and do

not contain viral nucleic acid.

Thus in a first aspect, the present invention provides a nucleic acid construct including a sequence encoding a  
5 plant viral coat protein containing a foreign or heterologous (the terms are used interchangeably) peptide insert, wherein the coat protein has been modified such as to reduce its ability to effect nucleic acid packaging within viral particles comprising said coat protein.

10 Preferably the modification will be such that the protein lacks its natural C-terminus. As discussed above, the nucleic acid may:

(i) be truncated such as to encode a protein truncated at  
15 its C-terminus,  
(ii) comprise a stop codon such as to encode a protein truncated at its C-terminus, or  
(iii) encode a coat protein comprising alterations to amino acid residues (e.g. deletion or substitution of  
20 positively charged residues) in the C-terminal region which effect nucleic acid packaging within viral particles comprising said coat protein.

By "natural" c-terminus is meant the C-terminus of the  
25 full-length coat protein expressed by the wild-type virus i.e. prior to any proteolytic degradation. The "C-terminal region" is that region which is lost during the interconversion of the relevant coat protein between different electrophoretic forms in nature, as described  
30 above.

Expression of the encoded chimaeric coat protein in an appropriate plant host along with other components required for assembly and/or replication of a plant viral  
35 particle (which may be provided within a modified viral genome or in trans) results in production of predominantly nucleic acid-free particles, generally at

least about 55-70% nucleic acid-free particles out of the total population of viral particles produced, preferably at least about 60% nucleic acid-free particles, more preferably about 70%, e.g. about 73%. This compares with  
5 very low levels of nucleic acid-free particles, such as 10% or less, generally around 5% and sometimes undetectable, on expression with full-length S capsid protein including the wild-type C-terminus.

10 The present invention also provides a composition including nucleic acid encoding capsid proteins of a plant virus, at least one of which contains a heterologous peptide insert, wherein one of the capsid  
15 proteins has been modified such as to reduce its ability to effect nucleic acid packaging within viral particles comprising said coat protein. Preferably the composition comprises a Small or S capsid protein lacking its natural C-terminus. The capsid protein containing the peptide  
20 insert may be the Small or S capsid protein (e.g. VP23 of a comovirus) or may be the Large or L capsid protein (e.g. VP37 of a comovirus). Nucleic acid encoding first and second capsid proteins of the plant virus may be included in the same nucleic acid molecule (indeed they  
25 may be encoded on a single ORF, the expression product of which is subsequently cleaved) which may be a vector and may be a modified plant-viral genome.

The composition, e.g. modified viral or plant genome, may include sequences encoding other components required for  
30 assembly of viral particles displaying the peptide insert on their surface. Again these may be on the same or separate nucleic acids (e.g. the composition may comprise a pair of plasmids).

35 A further aspect of the present invention provides a host cell harbouring such nucleic acid or modified plant viral genome, particularly a plant cell which may be comprised

in a plant or part of a plant. The plant may be a legume. The nucleic acid may be RNA or DNA, such as cDNA. As discussed elsewhere herein, for ease of manipulation it may be advantageous to use a DNA  
5 construct given the relative instability of RNA and its susceptibility to ubiquitous RN'ases.

Another aspect of the present invention provides a method which includes introduction of nucleic acid or modified  
10 viral genome according to the present invention into a host cell, such as a plant cell, which may be comprised in a plant or part of a plant. This may be by inoculation of the nucleic acid onto a plant or a part thereof. Subsequent expression from the nucleic acid  
15 results in production of viral particles displaying on their surface the peptide insert containing within the chimaeric viral capsid protein. Thus, plants or parts of plants including cells harbouring nucleic acid according to the present invention may be culture under appropriate  
20 conditions for production of viral particles by expression from the nucleic acid. Viral particles in a given population will be predominantly nucleic acid-free, i.e. a majority of the particles produced will lack nucleic acid, as discussed.

25 The present invention also provides a plant including a plant cell as disclosed, and part of the plant comprising the modified nucleic acids and/or particles of the present invention.

30 A plant according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It  
35 is noted that a plant need not be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant

or an ancestor thereof.

Suitable plants for use in production of a plant viral particle in accordance with the present invention include  
5 members of the *Leguminosae* (legumes) particularly *Vigna unguiculata* (cowpea or blackeye bean), also, *Phaseolus vulgaris*, other beans such as French bean, broad bean and soy bean, lupin and other plants such as *Solanaceae*,  
including tobacco plants such as *Nicotiana benthamiana*  
10 (For reference see for example "Viruses Infecting Forage Legumes", 1986, Monograph number 14, Edwardson and Christie, University of Florida, Gainesville, USA, including Chapter 6, Table 11, pages 114-115.)

15 The present invention also provides the use of nucleic acid or a modified viral genome, as disclosed, for the production of plant viral particles. The particles display a peptide insert at their surface. Particles of interest lack, i.e. do not contain, nucleic acid. The  
20 production generally takes place in a plant, following inoculation with the construct or modified viral genome.

Particles may be isolated by the method of van Kammen and de Jager. The method involves homogenisation of plant  
25 tissue and purification of the virus particles by polyethylene glycol precipitation and differential centrifugation from a plant in accordance with the present invention. Particles lacking nucleic acid may be isolated and/or purified and separated from particles  
30 which include nucleic acid by the caesium chloride centrifugation protocol which is standard in the art (van Kammen and de Jager (1978), Cowpea mosaic virus, *CMI/AAB Descriptions of Plant Viruses*, 197). Other methods for separating particles, such as are known to those skilled  
35 in the art, may equally be used.

Isolation may be followed by one or more steps of

purification, for instance using a binding molecule such as an antibody specific for the viral particles and/or the displayed peptide insert.

5      Following isolation, purification and/or separation, particles lacking nucleic acid may be formulated into a composition which includes at least one additional component, such as a pharmaceutically acceptable excipient, diluent, carrier or vehicle. Such a  
10      composition may include an adjuvant.

A composition according to a further aspect of the present invention, such as a pharmaceutically acceptable composition, includes plant viral particles containing a  
15      foreign or heterologous peptide insert within a capsid protein, and having a modified capsid protein such that they lack nucleic acid. Such particles may be substantially pure, preferably greater than about 90% particles which are nucleic acid free, more preferably  
20      greater than about 95% particles which are nucleic acid free, more preferably greater than about 98% particles which are nucleic acid free and most preferably substantially all particles which are nucleic acid free. That is to say that such compositions may contain an  
25      active ingredient which consists essentially of plant viral particles which display a foreign epitope on their surface and do not contain nucleic acid.

A still further aspect of the present invention provides  
30      a population of plant viral particles consisting essentially of plant viral particles displaying a foreign epitope and not containing nucleic acid. As discussed, production of viral particles in which the Small of "S" capsid protein lacks its natural C-terminus results in a  
35      population of particles which are predominantly nucleic acid free and which can be separated from particles containing nucleic acid, e.g. by caesium chloride

centrifugation.

Another aspect of the present invention provides a plant viral particle or a population thereof obtainable or  
5 obtained in accordance with the present invention as disclosed. Such particles may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other particles (e.g. lacking the  
10 modified coat proteins, or containing nucleic acids). Where used herein, the term "isolated" encompasses all of these possibilities. In particular such a population of modified plant virus particles will differ from those obtainable by prior art techniques in that they will  
15 uniformly comprise a coat protein which has been specifically modified such as to reduce its ability to effect nucleic acid packaging within the viral particles (i.e. not merely formed as a distribution of variably cleaved proteins) and will also be predominantly nucleic  
20 acid-free.

Interestingly, a 3.5Å electron density map of CPMV shows that there is a clear relationship between CPMV and the T=3 plant viruses such as the tombusviruses, in  
25 particular tomato bushy stunt (TBSV) and the sobemoviruses, in particular southern bean mosaic (SBMV). The capsids of these latter viruses are composed of 180 identical coat protein subunits, each consisting of a single  $\beta$ -barrel domain. These can occupy three different  
30 positions, A, B and C, within the virions. The two coat proteins of CPMV were shown to consist of three distinct  $\beta$ -barrel domains, two being derived from VP37 and one from VP23. Thus, in common with the T=3 viruses, each CPMV particle is made up of 180  $\beta$ -barrel structures. The  
35 single domain from VP23 occupies a position analogous to that of the A type subunits of TBSV and SBMV, whereas, the N- and C- terminal domains of VP37 occupy the



positions of the C and B type subunits respectively.

It is thus clear that the present invention may be applied to viruses over and above the comoviruses  
5 discussed in detail below.

Various embodiments of the present invention will now be discussed in more detail.

10 A suitable insertion site for the foreign insert (e.g. epitope) may be found by identifying that part of the virus genome which encodes an exposed portion of a coat protein. Within this part of the genome two different  
15 restriction enzyme sites are chosen and the nucleic acid cleaved using the appropriate restriction enzymes. Pairs of complementary oligonucleotides are synthesised encoding the foreign peptide which it is desired to insert into the virus coat protein. The oligonucleotides terminate in ends which are compatible with the  
20 restriction enzyme sites thus allowing insertion into the cleaved virus nucleic acid. This procedure results in the introduction of a nucleotide sequence coding for a foreign peptide whilst avoiding the presence of direct sequence repeats flanking the insert. Preferably  
25 complementary oligonucleotides are synthesised in which the sequence encoding the heterologous amino acids are flanked by plant virus-specific sequences so that the foreign nucleic acid is inserted as an addition to the existing nucleic acid.

30 The three dimensional structure of a plant virus may be examined in order to identify portions of a coat protein which are particularly exposed on the surface, and which have little secondary structure and are therefore  
35 unlikely to be involved in inter-subunit interactions. These are potentially optimum sites for insertion. For instance the amino acid sequence of the exposed portions

of a coat protein may be examined for amino acids which break  $\alpha$ -helical structures. Examples of suitable amino acids are proline and hydroxyproline, both of which whenever they occur in a polypeptide chain interrupt the  $\alpha$  helix and create a rigid kink or bend in the structure. Other target residues having the desired properties may be recognised by those skilled in the art e.g. with the assistance of computer-modelling techniques.

To demonstrate the system, the plant virus cowpea mosaic comovirus (CPMV) was chosen. The three-dimensional structure of CPMV has been solved at atomic resolution which has enabled identification of sites suitable for modification without disruption of the particle structure.

CPMV is a bipartite RNA virus and in order to manipulate the genome of any RNA virus to express foreign peptides it is advantageous to use cDNA clones of the RNA. Full length cDNA clones of both CPMV RNA molecules are available (Dessens & Lomonossoff, (1991). Virology 184, 738-746; Rohll et al., (1993), Virology 193, 672-679) which can be manipulated to insert oligonucleotide sequences encoding a foreign peptide. cDNA clones of the genome from plant RNA viruses can be used to generate *in vitro* transcripts that are infectious when inoculated onto plants. However, the infectivity of the transcripts is significantly lower than that of natural virion RNAs, probably as a result of the presence of non-viral residues at the termini of the transcripts. Difficulties may also be caused by exposure of the transcripts to degradative agents during inoculation. For this reason the transcripts are usually stabilised by capping their 5' ends, but this is an inefficient, costly and time-consuming process.

cDNA clones of CPMV RNAs M and B have been constructed,

in which the cDNA clone of the M RNA contains an inserted oligonucleotide sequence encoding a foreign peptide, which make use of the cauliflower mosaic virus (CaMV) 35S promoter sequence linked to the 5' ends of the viral cDNAs to generate infectious transcripts in the plant. This technique overcomes some of the problems encountered with the use of transcripts generated *in vitro* and is applicable to all plant RNA viruses (Dessens & Lomonossoff, (1993), Journal of General Virology 74, 889-892).

Those skilled in the art are well able to construct vectors and design suitable protocols, e.g. in view of, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press.

For instance, the skilled person will include a suitable promoter in a DNA construct. The function of the promoter in a DNA construct is to ensure that the DNA is transcribed into RNA containing the viral sequences. By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). A promoter "drives" transcription of an operably linked sequence.

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter, or "in functional combination" therewith.

Preferred promoters include the 35S promoter of cauliflower mosaic virus or the nopaline synthase promoter of *Agrobacterium tumefaciens* (Sanders, P. R., et

al (1987), *Nucleic Acids Res.*, 15: 1543-1558). These promoters are active in many, if not all, cell types of many plants.

5 For making *in vitro* transcripts, virus-specific DNA may be operably linked downstream of bacteriophage T7, T3 or SP6 promoters, for example.

10 Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis (e.g. of residues responsible for nucleic acid packaging), sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Protocols in Molecular*  
15 *Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

Specific procedures and vectors previously used with wide success upon plants are described by Bevan, *Nucl. Acids*  
20 *Res.* (1984) 12, 8711-8721), and Guerineau and Mullineaux, (1993) *Plant transformation and expression vectors*. In: *Plant Molecular Biology Labfax* (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. In addition to the regenerated plant, the present invention embraces all of  
25 the following: a clone of such a plant, seed, selfed or hybrid progeny and descendants (e.g. F1 and F2 descendants) and any part of any of these. The invention also provides a plant propagule from such a plant, that is any part which may be used in reproduction or  
30 propagation, sexual or asexual, including cuttings, seed and so on.

In a preferred embodiment the plant virus is a comovirus such as cowpea mosaic virus (CPMV). Preferably, the  
35 foreign insert is made immediately preceding a proline residue in the  $\beta$ B- $\beta$ C loop of the small capsid protein (VP23), which in CPMV is the proline 23 (Pro<sup>23</sup>).

In the inventors' original HRV-14 chimaera (called LO) the epitopes were inserted between alanine 22 and proline 23 of the S protein of CPMV (Figure 3). Numerous peptides have been inserted at this position and in all cases these undergo a cleavage reaction between the last two residues of the epitope (lysine 1097 and leucine 1098 in the HRV-14 LO example tested). Thus the epitope is presented on the surface of CPMV as a linear peptide rather than as a constrained loop. The cleavage reaction has been characterised by a combination of polyacrylamide gel electrophoresis (SDS-PAGE), crystallography and protein sequence data.

In a further preferred embodiment, the insert does not cleave, e.g. by virtue of the insert being one or more amino acids away from a particular proline residue in the  $\beta$ B- $\beta$ C loop of the small capsid protein, which in CPMV may advantageously be one amino acid to the left in the  $\beta$ B- $\beta$ C loop of the small capsid protein (VP23) of CPMV (Figure 3), i.e. between proline 21 and alanine 22.

Generally, the peptide insert includes one or more epitopes, such as an epitope of a pathogen to which it is advantageous or desirable to provoke an immune response, such as raising antibodies, in a mammal. This may have a prophylactic and/or therapeutic aim, as discussed below, or may be in order to provide for an antibody titre against an epitope of interest in the serum of a mammal to allow for isolation of the antibody from the mammal (optionally following a step of sacrificing of the mammal), e.g. for use in generation of monoclonal antibodies and/or use in various in vitro or in vivo applications of antibodies which are well known to those skilled in the art.

The wide applicability of peptide display on the surface of plant viruses has been demonstrated using antigenic

peptides from a number of different animal viruses and bacterial pathogens of animals, and also using at least one mammalian peptide hormone were used. Attention is drawn once again to WO92/18618 and WO96/02649, and the experimental results included below. Two of the viruses exemplified belong to the picornavirus group of animal viruses - foot and mouth disease virus (FMDV) and human rhinovirus (HRV). There are several important pathogens in this group, particularly, FMDV, poliomyelitis (polio) and hepatitis A. Another virus selected was human immune deficiency virus (HIV) which bears no similarity to any known plant virus, and for which no successful vaccines are currently available. The bacterial pathogen was *Staphylococcus aureus*, a causative agent of several animals diseases including mastitis in cows. The peptide hormone was porcine gonadotrophin releasing hormone.

For applications in raising an immune response to a peptide insert, the present invention provides an especially attractive epitope presentation system. When used for such applications the antigenic peptide component will be sited appropriately on the virus particle so as to be easily recognised by the immune system, for example by location on an exposed part of the coat protein of the virus. As applied to the latter, therefore, the invention provides assembled particles of a modified plant virus containing an epitope derived from a pathogen, e.g. an animal virus, incorporated in an exposed position on the surface of the coat protein of the plant virus. This invention also encompasses the use of such assembled modified plant virus particles as the immunogenic component of a composition, preferably a pharmaceutically acceptable composition, and in the manufacture of a medicament for administration to a mammal, e.g. for raising an immune response to the peptide insert, which immune response may include an antibody response. Such assembled modified plant virus

particles presenting antigenic peptides also have applications as the antigen presentation component of an immunodiagnostic assay for detection of e.g. animal (including human) pathogens and diseases.

5

Pathogens of which peptides, including peptides which include one or more epitopes, may usefully be inserted into a plant viral capsid protein for display or presentation on the surface of a plant viral particle, lacking nucleic acid in accordance with the present invention, include bacteria such as *Corynebacteria*, *Pneumococci*, *Streptococci* such as *S. pyrogenes*, *Staphylococci* such as *S. aureus*, *Enterobacteriaceae* including *E. coli*, *Salmonellae* such as *S. typhimurium*, and *Shigellae* such as *S. dysenteriae*, *Vibrio cholerae*, *Hemophilus-Bordetella* bacteria including *Hemophilus influenza* and *B. pertussis*, spore-forming *Bacilli* such as *B. anthracis* and *B. subtilis*, *Clostridium botulinum* and *Clostridium tetani*, *Mycobacterium* such as *M. tuberculosis*, *M. leprae* and *M. bovis*, *Actinomycetes* and *Spirochetes*, also trypanosomes, mycoplasma, such as *Aspergillus nidulans*, and other parasites and fungi, including *Plasmodium*, *Candida albicans*, *Rickettsiae* and *Chlamydia*, also viruses such as herpes viruses, pox viruses, picornaviruses such as poliovirus and rhinoviruses, myxoviruses including Influenza (A, B and C), mumps, measles and rubella viruses, also Respiratory Syncytial Virus, Canine Distemper Virus, Rinderpest Virus, Yellow Fever Virus, Dengue Virus, Semliki Forest Virus, CMV, and reoviruses, retroviruses including HIV I and II, HTLV I and II, hepatitis viruses including Hepatitis A, B and C Viruses, and tumour viruses such as Maloney Leukaemia Virus and Human and Bovine Papilloma Viruses. Peptide fragments of a number of these and others have already been displayed or presented on the surface of plant viruses, as discussed herein.

35

A pharmaceutically acceptable composition according to the present invention may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or one or more other materials well known to those skilled in the art. Such materials should generally be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

Also envisaged is use of (edible) plant material in accordance with the present invention in raw or unprocessed form e.g. the edible parts of plants having relatively high concentrations of the recombinant capsid particles therein, and relatively low concentrations of the viral nucleic acid.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection,



Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

5 Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount  
10 administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically  
15 takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's  
20 Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

Further aspects of the present invention and embodiments thereof will be apparent to those skilled in the art.  
25

The present invention will now be exemplified with reference to following experimental examples and the figures discussed already above.

30 Brief Description of the Figures

Figure 1 shows the three-component nature of comoviruses. The protein and RNA contents of the components are indicated.

35 Figure 2 shows the sequence of the C-terminus of the S protein of CPMV showing the position of the premature termination in the SM4 mutant and truncation in the DM4

mutant, subject of experiments described in Example 1. In SM4 the codon encoding the amino acid residue marked with an asterisk was mutated to a stop codon, while in DM4 nucleotides encoding all amino acids following the  
5 arrow were deleted from the coding sequence. The arrow marks the site of natural proteolytic cleavage of the CPMV S protein.

Figure 3 shows the position of the HRV epitope (HRV-LO -  
10 Figure 3(a)) within the  $\beta$ B- $\beta$ C loop of the small (S) coat protein of CPMV and the construct HRV-L1 (Figure 3(b)) in which the HRV epitope has been moved one amino acid upstream, as described in Example 2. The HRV epitope is in bold.

15 Figure 4 shows results of ELISA on antisera raised in rabbits against CPMV wt and the CPMV/HRV chimaeras, HRV-L0 and HRV-L1, as described in Example 2. The sera were assessed for their ability to bind to virus particles of  
20 native HRV14 in an ACP (antigen coated plate)-ELISA in which HRV-14 was adsorbed directly to the wells of ELISA plates.

Figure 5 shows the specific infectivities of DM4 and  
25 wild-type CPMV.

### Examples

#### EXAMPLE 1

*Increasing the Proportion of Empty Particles in a CPMV  
30 Preparation*

Cowpea mosaic virus (CPMV) is the type member of the comovirus group of plant viruses. As noted, it has a bipartite RNA genome separately encapsidated within icosahedral particles, which are composed of sixty copies  
35 each of a large and a small coat protein.

Preparations of comoviruses, including CPMV, can be

separated into three components designated Top(T) middle (M) and bottom (B) by centrifugation on density gradients. The three components have identical protein composition but differ in the RNA content of the capsids. T components are devoid of RNA, B components contain RNA 1 of the CPMV genome, M components contain RNA 2. This is summarised in Figure 1.

A deletion mutant was made in which the C-Terminal 24 residues of the small coat protein of CPMV were removed.

A premature stop codon was introduced into an infectious cDNA clone of CPMV RNA-2 at the natural cleavage site of the S protein, as shown in Figure 2. This mutant was designated "SM4". Cowpea (*Vigna unguiculata*) plants were inoculated in the presence of a cDNA clone of RNA-1.

All plants showed primary symptoms 21 days post DNA inoculation, whereas only a third showed systemic symptoms. Lesions on inoculated leaves were smaller than wild-type and were surrounded by necrotic rings. The infections could be mechanically transmitted to healthy plants which developed wild-type symptoms on both inoculated and secondary leaves. All plants showed both primary and systemic symptoms 14 days post passage inoculation. SDS-PAGE analysis of virus purified from first passage plants revealed that in all cases the S protein had reverted to the size of the wild-type in systemically infected leaves, reversion which was further confirmed by RT-PCR sequencing of the viral RNA.

To overcome the reversion with the site-directed mutant, a deletion mutant DM4 was made, truncated at amino acid 189 of the S protein. On inoculation, all cowpea plants showed primary symptoms, with small lesions surrounded by necrotic rings, whereas none showed systemic symptoms. On passaging, the infection maintained its phenotype on

the inoculated leaves, and systemic movement was significantly delayed compared with wild-type. The small lesion phenotype was also observed on *Phaseolus vulgaris*, a local lesion host of CPMV. SDS-PAGE analysis of virus purified from first passage cowpea plants showed no sign of reversion of the S protein, and this was confirmed by RT-PCR analysis.

DM4 virus was also extracted from plants and analysed by analytical ultra-centrifugation, a standard technique which enables the calculation of the relative amounts of T, M and B components. The deleted virus (DM4) showed a large increase in the proportion of T component in comparison with wild-type CPMV. Astonishingly this was over 70% in DM4 compared with a non-detectable level in the wild-type CPMV. The results are shown in Table 1.

The inventors repetition of these experiments using the same protocol has shown consistently the production of about 50-70% T component out of the total T, M and B components.

This was wholly unexpected. Although, the increased proportion of capsids lacking nucleic acid in DM4 probably explains the later onset of symptoms and the small lesion phenotype, the inventors initial explanation of these symptoms and phenotype was that the cleavable C-terminus of the S protein is involved in the spread of CPMV, with the presence or absence of the C-terminus being important in controlling the host response to infection. Such explanation teaches away from utility of truncated S protein and viral particles including it in the context of production of antigenic and/or immunogenic compositions, e.g. because of discouragement over perceived likely yield. However, given the additional results on nucleic acid incorporation into particles, the effect of the C-terminal deletion appears to be on

encapsidation rather than movement as was initially thought.

5 Thus, creation of plant virus particles in which the small coat protein lacks the natural C-terminus results in a large increase in the proportion of empty capsids (lacking nucleic acid). Insertion of epitopes into the truncated form of the coat protein should produce a large proportion of empty capsids displaying foreign peptide on  
10 the surface. The empty capsids (top component) may be separated from the middle and bottom components by centrifugation and used as a nucleic acid-free epitope presentation system, as discussed.

15 An additional benefit of using such an approach as the basis for chimaeras, is that the pattern of bands on SDS-PAGE gels is simpler, there being only one form of the small coat protein as opposed to multiple forms in the wild-type (i.e. expressing a full-length small coat  
20 protein) resulting from proteolytic cleavage of the C-Terminus of the wild-type small coat protein. This simpler and more consistent pattern is desirable from a quality-control point of view.

25 *EXAMPLE 2*

*Alternative Insertion Site In CPMV - Non-cleavage Of The Insert Epitope*

A construct in which HRV epitopes are inserted between the proline 21 and alanine 22 of the  $\beta$ B- $\beta$ C loop of the  
30 small (S) coat protein of CPMV (see Figure 3), designated HRV-L1, was produced as described in Porta et al., (1994) and inoculated onto cowpea plants. Following infection, chimaeric virus particles were purified and the coat proteins separated by SDS-PAGE.

35

Bands on an SDS-PAGE gel of the HRV-L1 virus particles compared with the original construct HRV-L0 clearly show

the absence of the cleavage product in L1.

HRV-L1 virus particles were incubated at 37°C over a two week period and samples removed every other day and  
5 analysed by SDS-PAGE. After 2 weeks at 37°C there was no evidence of the cleavage product in HRV-L1, indicating that the loop structure of the chimaera is stable.

Antisera were raised in rabbits against CPMV wt and the  
10 CPMV/HRV chimaeras, HRV-L0 and HRV-L1. The sera were assessed for their ability to bind to virus particles of native HRV14 in an ACP (antigen coated plate)-ELISA in which HRV-14 was adsorbed directly to the wells of ELISA plates. A range of dilutions of the different antisera  
15 were allowed to react with the immobilised virus particles. The binding of the rabbit antisera was revealed with goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase, followed by the addition of the substrate para-nitrophenyl phosphate (PNPP). The results  
20 in Figure 4, show that the anti HRV-L0 sera gave levels of reactivity with HRV-14 not significantly different to those obtained with a serum raised against wt CPMV. In contrast, the anti-HRV-L1 sera produced readings which were significantly superior to the background readings  
25 obtained with preimmune antiserum and about 5 times higher than those obtained with the anti-HRV-L0 sera.

This can be attributed to the fact that the HRV epitope probably adopts a structure similar to its counterpart in  
30 native HRV14 when presented as a closed loop in HRV-L1 as opposed to the cleaved form in HRV-L0.

#### TABLE 1

A comparison of the proportions of Top, Middle and Bottom  
35 components in DM4 and wild-type CPMV virus preparations.

"N.D." = not detectable.

	<u>TOP</u>	<u>MIDDLE</u>	<u>BOTTOM</u>
<u>DM4</u> :	73%	16%	11%
<u>WILD-TYPE</u> :	N.D.	40%	60%

### EXAMPLE 3

5

#### *Comparison of infectivity of deletion mutant and wild-type CPMV*

A local lesion assay was carried out to compare the proportion of infectious units (nucleic acid-containing particles) in the mutant DM4 (having a 24 amino acid C-terminal deletion) and wild-type virus preparations.

Such specific infectivity assays are performed on the local lesion host, *Phaseolus vulgaris* (French bean). On this host the wild-type CPMV does not spread to the upper leaves and the number of lesions per leaf or half-leaf corresponds to the number of infectious units, up to the point at which the dose response is saturated.

As DM4 has a large proportion of empty capsids it would be expected that if virus preparations which had equal protein content were compared the DM4 preparation would have fewer infectious units than wild-type.

Opposite half leaves were inoculated with either DM4 or a wild-type virus preparation at a particular protein concentration (Protein concentration was determined using the Bradford assay). The following protein concentrations were used; 0.4, 0.04, 0.004, 0.0004 mg/ml protein.

The number of lesions per half leaf was counted. The mean no. of lesions per half leaf is plotted (Figure 5), error bars represent the SEM. The dose response is probably saturated at 0.4 mg/ml while at lower concentrations the DM4 preparations were estimated to contain 6-fold fewer

infectious units when compared with wild-type on an equal protein basis. These results are shown in tabular form below:

5      *Comparison of the specific infectivities of DM4 and wild-type virus preparations when inoculated on an equal protein basis*

10	Inoculum concentration (mg/ml protein)	DM4 Mean number lesions/half leaf	WT Mean number lesions/half leaf	Mean lesions (WT) Mean lesions (DM4)
	0.4	76	200	2.6
	0.04	18.2	140	7.6
15	0.004	7.4	46.6	6.2
	0.0004	---	5.6	N/A

Key: --- No symptoms at 14 d.p.i

20

#### EXAMPLE 4

25      *Infectivity of DM4 virus preparations in cowpea plants*  
CPMV produces a systemic infection (the inoculated and the upper (trifoliate) leaves become infected) on its natural host *Vigna unguiculata* (cowpea). Cowpea is therefore a useful host for propagating virus particles e.g. for vaccine purposes.

30

Plants were inoculated with either the mutant DM4 or the wild-type. Either 1 or 10 µg/ml of purified virus was used as the inoculum.

35

At 1 µg/ml the appearance of symptoms on the primary (inoculated) leaves of DM4-inoculated plants was delayed



by 2-7 days compared with wild-type (ie the minimum delay was 2 days and the maximum delay 7 days). No symptoms were observed on the trifoliate (upper) leaves at this concentration.

5

At 10  $\mu$ g/ml the appearance of symptoms on the primary (inoculated) leaves of DM4 inoculated plants was delayed by 2-5 days compared with wild-type. Symptoms appeared on the trifoliate (upper leaves) 9-14 days after wild-type. These symptoms had the same mosaic appearance as wild-type symptoms.

10

This illustrates that systemic infection is achievable with the DM4 construct on cowpea plants, albeit that a higher inoculum dose is required to produce systemic infection. It is thus clear that the nucleic acids of the present invention may be readily employed to produce practical quantities of particles e.g. for vaccine purposes even though these are substantially nucleic acid-free.

15

20

Claims

1. A nucleic acid construct comprising a sequence encoding a plant viral coat protein containing a foreign or heterologous peptide insert, characterised in that  
5 said coat protein has been modified such as to reduce its ability to effect nucleic acid packaging within viral particles comprising said coat protein.
2. A nucleic acid as claimed in claim 1 wherein the  
10 encoded coat protein lacks its natural C-terminus.
3. A nucleic acid as claimed in claim 2 wherein the nucleic acid sequence:  
(a) is truncated such as to encode a protein truncated at  
15 its C-terminus,  
(b) comprises a stop codon such as to encode a protein truncated at its C-terminus, or  
(c) encodes a coat protein comprising alterations to amino acid residues in the C-terminal region which effect  
20 nucleic acid packaging within viral particles comprising said coat protein.
4. A nucleic acid as claimed in claim 3(c) wherein the alterations to amino acid residues comprise substitution  
25 or deletion of positively charged amino acids in the C-terminal region.
5. A nucleic acid as claimed in any one of the preceding claims wherein the coat protein is derived from  
30 a comovirus or a T=3 plant virus.
6. A nucleic acid as claimed in claim 5 wherein the coat protein is derived from cowpea mosaic virus.
- 35 7. A nucleic acid as claimed in claim 6 wherein the coat protein is the small capsid protein of cowpea mosaic virus.

8. A nucleic acid as claimed in claim 7 wherein the heterologous insert is made in the  $\beta$ B- $\beta$ C loop of the small capsid protein.
- 5 9. A nucleic acid as claimed in claim 8 wherein the heterologous insert is made immediately preceding proline 23 of the small capsid protein.
- 10 10. A nucleic acid as claimed in claim 8 wherein the heterologous insert is made one amino acid away from proline 23 of the small capsid protein.
- 15 11. A nucleic acid as claimed in any one of the preceding claims which is a modified viral or plant genome.
- 20 12. A nucleic acid as claimed in any one of the preceding claims wherein the sequence encoding the coat protein is operably linked to a promoter.
- 25 13. A nucleic acid composition comprising a nucleic acid as claimed in any one of the preceding claims and further comprising sequences encoding other components required for assembly and/or replication of viral particles displaying the peptide insert on their surface.
- 30 14. A nucleic acid composition comprising nucleic acid sequences encoding two or more capsid proteins of a plant virus, at least one of which contains a heterologous peptide insert, wherein one of the capsid proteins has been modified such as to reduce its ability to effect nucleic acid packaging within viral particles comprising said coat protein.
- 35 15. A nucleic acid composition as claimed in claim 14 wherein the encoded capsid proteins are the small and large capsid proteins of a comovirus, and wherein said

small capsid protein lacks its natural C-terminus.

5 16. A nucleic acid composition as claimed in any one of claims 13 to 15 wherein said all of said sequences are present on a single nucleic acid molecule.

10 17. Nucleic acid as claimed in any one of the preceding claims wherein the heterologous insert encodes any one or more of the following: a peptide hormone; an enzyme; a growth factor; an antibody; a cytokine; an epitope or antigen of protozoal, viral, bacterial, fungal or animal origin.

15 18. Nucleic acid as claimed in claim 17 wherein the heterologous insert encodes more than 5; about 6-10; 10-20; 20-30; or up to 38 amino acids.

20 19. Use of the nucleic acid of any one of the preceding claims for the production of plant viral particles which are predominantly nucleic acid-free and wherein the heterologous peptide insert is expressed on the surface of the viral capsid particles.

25 20. A method for the production of plant viral particles which includes the step of introducing nucleic acid of any one of claims 1 to 18 into a plant cell.

30 21. A method for the production of plant viral particles which comprises the step of causing or allowing the expression nucleic acid of any one of claims 1 to 18 in an appropriate plant host along with other components required for assembly of a plant viral particle.

35 22. A method as claimed in claim 21 wherein the particles are subsequently isolated from the plant host.

23. A host cell comprising nucleic acid of any one of

claims 1 to 18.

24. A host cell as claimed in claim 23 which is a plant  
cell and which is comprised within all or part of a  
5 plant.

25. A plant including the plant cell of claim 24.

26. A plant as claimed in claim 25 which is cowpea.  
10

27. A part of a plant as claimed in claim 25 or claim  
26, which part comprises modified plant viral particles  
which:

(i) comprise a coat protein which has been modified such  
15 as to reduce its ability to effect nucleic acid packaging  
within the viral particles,  
(ii) are predominantly nucleic acid-free,  
(iii) have a heterologous peptide insert expressed on the  
surface of the viral capsid particles.

20 28. A population of modified plant virus particles:  
(i) each of which comprises a coat protein which has been  
identically modified such as to reduce its ability to  
effect nucleic acid packaging within the viral particles,  
25 (ii) are predominantly nucleic acid-free,  
(iii) have a heterologous peptide insert expressed on the  
surface of the viral capsid particles.

29. A composition comprising the part of a plant, or the  
30 population of particles, of claim 27 or claim 28, plus at  
least one additional component.

30. A composition as claimed in claim 29 wherein the  
additional component is as a pharmaceutically acceptable  
35 excipient, diluent, carrier or vehicle, and/or adjuvant.

31. Use of the part of a plant, the population of

particles, or the composition of any one of claims 27 to 30 for raising an immune response to the peptide insert in mammal.

- 5      32. A method for providing an antibody titre against an epitope of interest in the serum of a mammal comprising the use of claim 31, optionally following a step of sacrificing of the mammal.

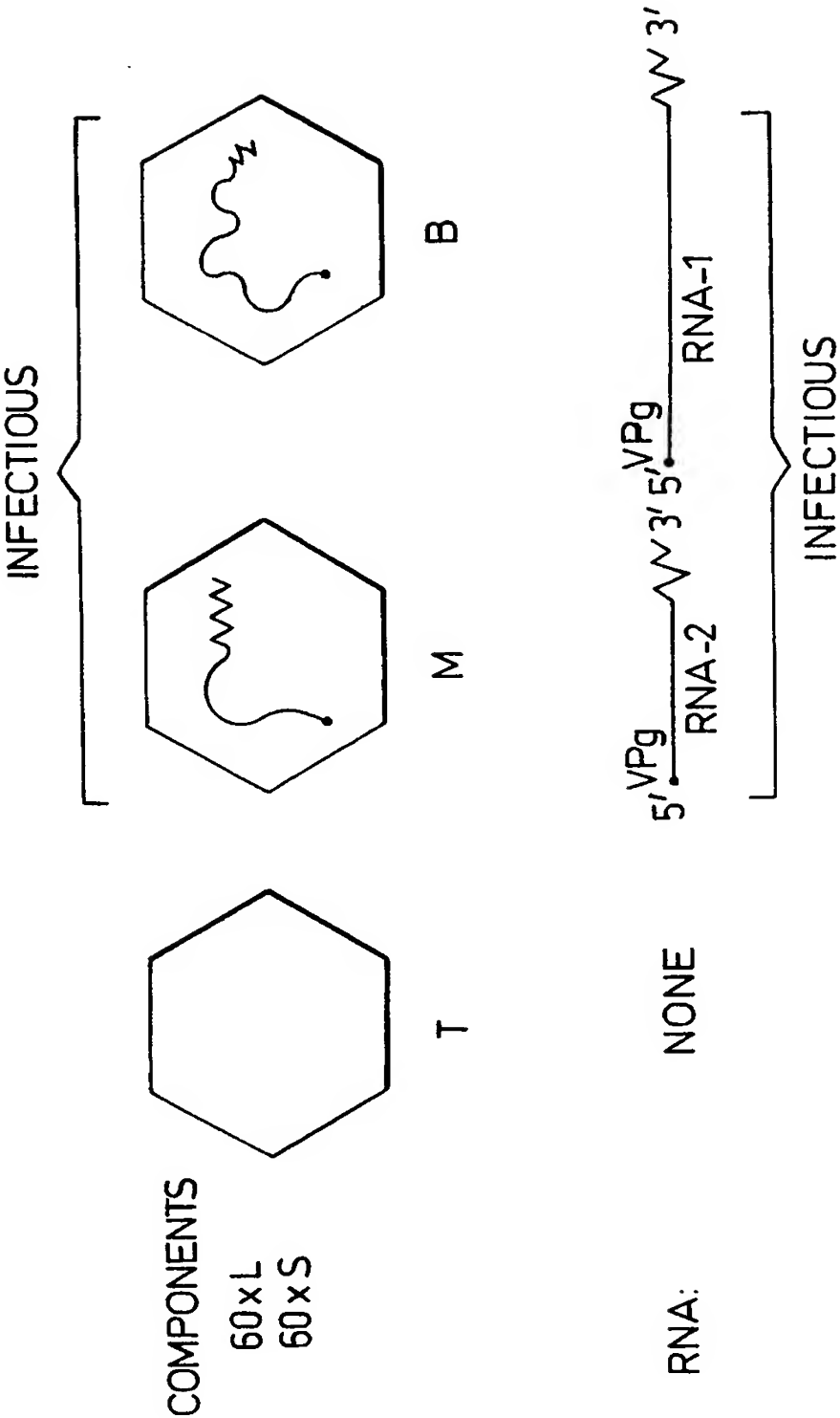


Fig. 1

2/5

-----βI-----→  
170 180 190 200 210  
VNMRFDPNFRVAGNIILMPFPPLSTETPPLLKFRFRDIERSKRSVMVGHTATAA  
↓\*

*Fig. 2*

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*Fig. 3*

S T P P A K D A T G I D N H R E A K L P F S D  
 CTAGCACTCCTCCTAAGATGCTACTGGAATCGAATCATAGAGAAGCAAAATTGCCATTTTCAGACGT  
 GTGAGGAGGACGATTTCTACGATGACCTTAGCTATTAGTATCTCTTCGTTTAAACGGTAAAGTC

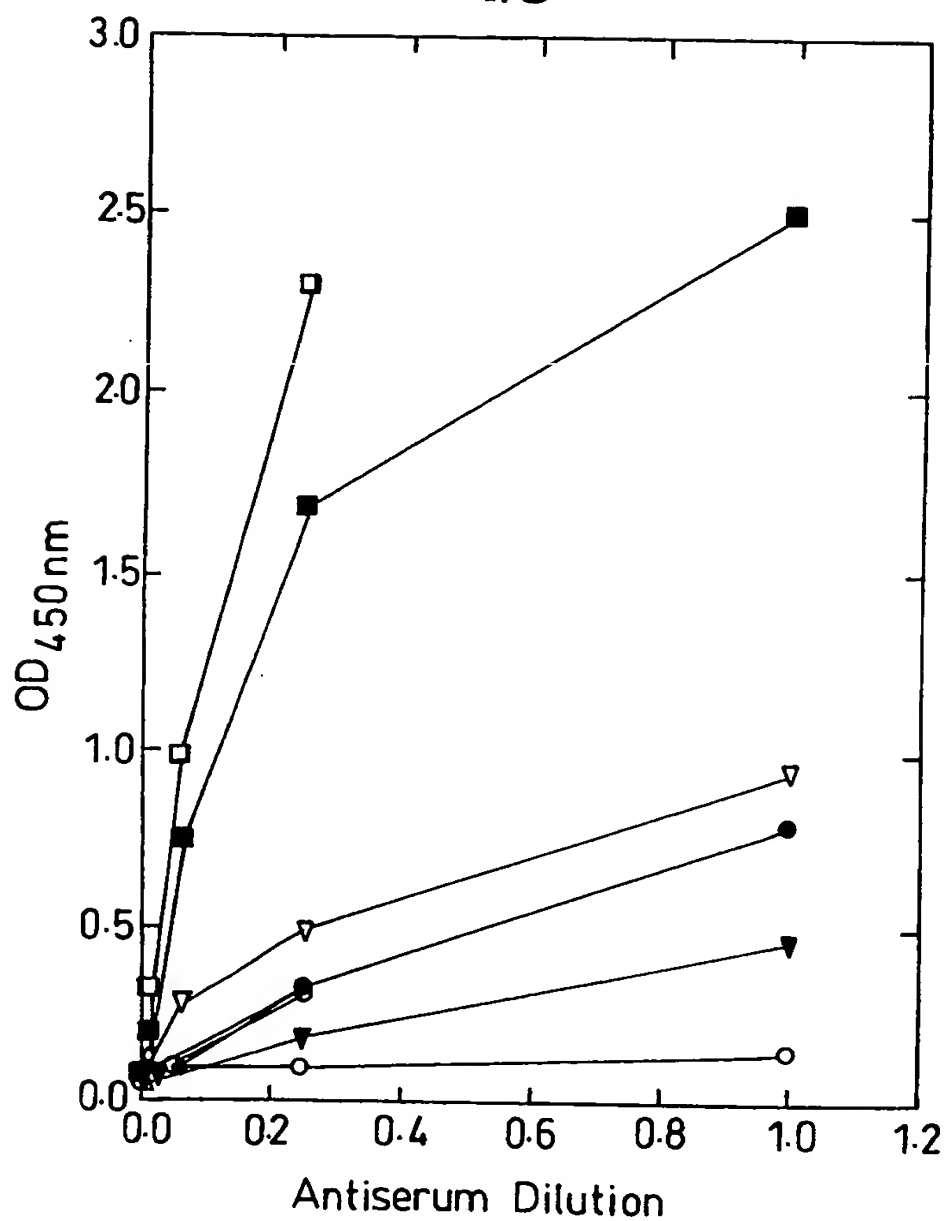
*(a)*

S T P P A K D A T G I D N H R E A K L A P F S D  
 CTAGCACTCCTCCTAAGATGCTACTGGAATCGAATCATAGAGAAGCAAAATTGGCTCCATTTTCAGACGT  
 GTGAGGAGGATTTCTACGATGACCTTAGCTATTAGTATCTCTTCGTTTAAACCGAGGTAAAGTC

*(b)*

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4/5

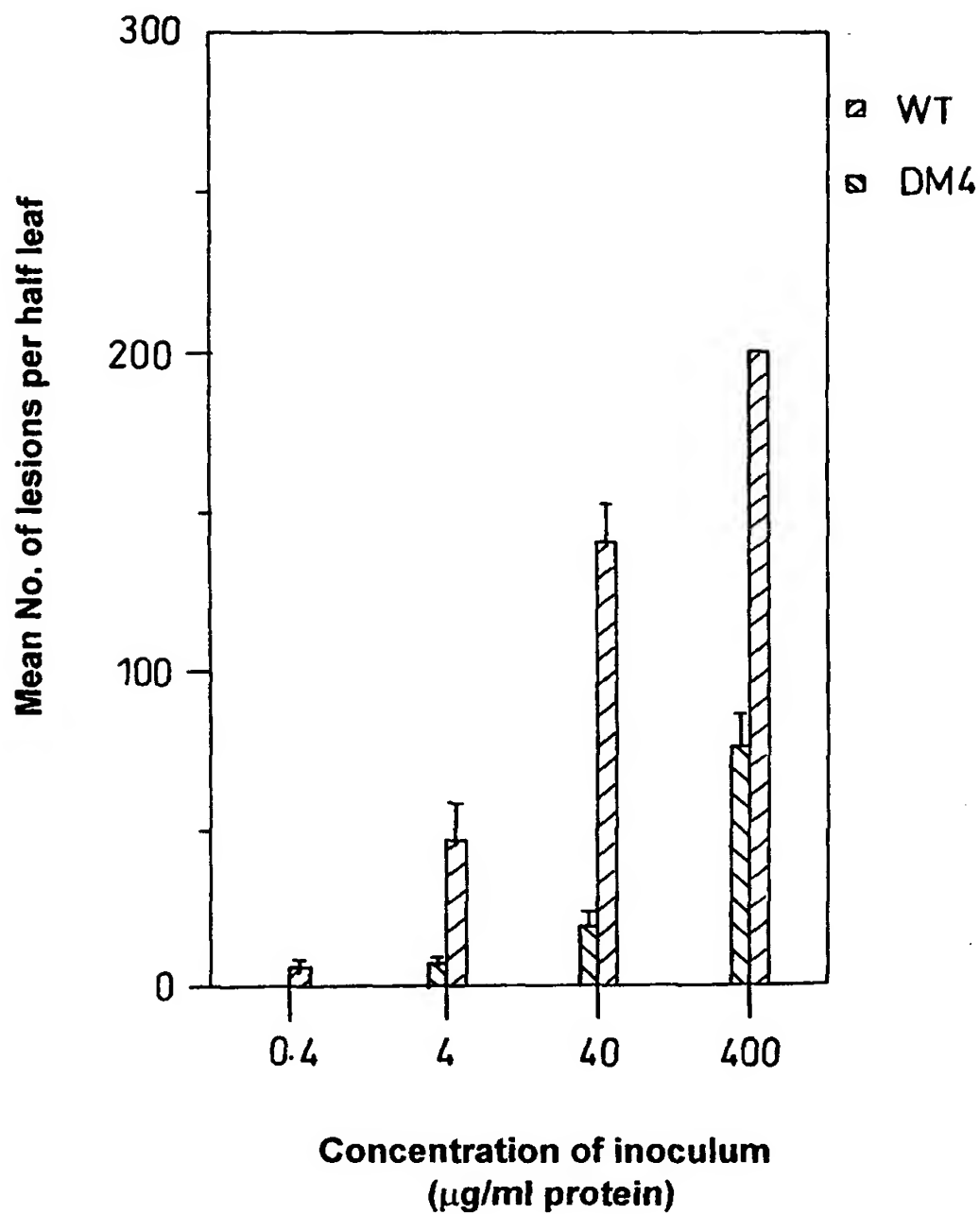
**Fig. 4**

- ○ — PRE IMMUNE SERA
- ● — α-WT-CPMV
- ▽ — α HRV LO RABBIT I
- ▼ — α HRV LO RABBIT II
- □ — α HRV L1 RABBIT I
- ■ — α HRV L1 RABBIT II

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**5/5**

**Specific infectivities of DM4 and wild-type CPMV  
When inoculated on an equal protein basis**



***Fig. 5***

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# INTERNATIONAL SEARCH REPORT

tional Application No

PCT/GB 98/01725

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/82 C12N15/41 C07K14/095 C12N7/04 A61K39/125

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHMITT, C., ET AL. : "in vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75kD readthrough protein is important for efficient virus assembly" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 89, July 1992, pages 5715-5719, XP002081569 see the whole document ---	1-3,8, 11-13, 16-32
Y	WO 96 02649 A (AXIS GENETICS LTD ; LOMONOSSOFF GEORGE PETER (GB)) 1 February 1996 cited in the application see the whole document ---	1-3,8, 11-13, 16-32
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

21 October 1998

Date of mailing of the international search report

02/11/1998

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Authorized officer

Holtorf, S

# INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/GB 98/01725

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DALSGAARD, K., ET AL. : "plant-derived vaccine protects target animals against a viral disease"</p> <p>NATURE BIOTECHNOLOGY, vol. 15, March 1997, pages 248-252, XP002081570 cited in the application see the whole document</p> <p style="text-align: center;">---</p>	1-32
A	<p>ALDOVINI, A., ET AL. : "mutations of RNA and protein sequences involved in human immunodeficiency virus type 1 packaging results in production of noninfectious virus"</p> <p>JOURNAL OF VIROLOGY, vol. 64, no. 5, May 1990, pages 1920-1926, XP002003496 abstract, page 1920; 1921, right column; page 1922, right column; page 1923-1925; Fig. 4</p> <p style="text-align: center;">-----</p>	1-32

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 98/01725

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 31 and 32  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

### Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. J. Application No

PCT/GB 98/01725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9602649 A	01-02-1996	AU 2893495 A	16-02-1996
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